Int.J.Curr.Microbiol.App.Sci (2025) *14*(06): 293-300

International Journal of Current Microbiology and Applied Sciences
***ISSN: 2319-7706* Volume 14 Number 6 (2025)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2025.1406.026

Cytopathological Changes in the Oral Mucosa of Tobacco Users in Ibadan, Nigeria

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Keywords

Tobacco, cytopathology, Papanicolau, oral cancer

Article Info

Received:
02 March 2025
Accepted:
09 May 2025
Available Online:
10 June 2025

A B S T R A C T

Tobacco use is an important risk factor in the cause of oral cancer, as it is the most popular preventable cause of death, hence, the need for members of the public to know how its consumption impact them negatively, so as to protect themselves from its dangers. This study was conducted to determine the cytopathological changes in the oral smears of tobacco users in Oyo state. A total of 205 participants, which consisted of 95 males and 45 females tobacco users, and 35 males and 30 females non-tobacco users (served as controls), were recruited for this study. Buccal smear specimen was collected on a clean, grease-free slide using Ayre's spatula. The slides were stained using Papanicolaou staining technique, and viewed with light microscope to examine their cytomorphological appearances. In addition, prevalence of different cellular morphological appearance in tobacco users and non-users was tested using chi-square. The p values at less than 0.05 were considered significant. The cytopathological features observed were increased micronuclei, binucleation, karyorrhexis, karyolysis, pyknosis, and perinuclear halo were observed in the oral smears of tobacco users when compared with the non-tobacco users. The occurrence of normal cells showed a very high value ($x^2 = 73.17$, $p = 0.0000$), indicating a highly significant difference between tobacco users and non-users, which suggests that normal cells are significantly less prevalent in tobacco users compared to the control group. However, cell types such as KH - Karyorrhesis (p = 0.030), PN - Pyknosis ($p = 0.007$), PH - Perinuclear Halo ($p = 0.006$), and BC - Binucleated Cell ($p = 0.025$) all exhibited statistically significant differences between tobacco users and non-users. This implies that these cell types are more prevalent in test group compared to the control, which suggest that while certain cell types, such as KH, PN, PH, and BC, are significantly affected by tobacco usage, others, like CG, KL, and MN do not show a statistically significant difference between tobacco users and non-users. It can be safely concluded that tobacco use greatly caused alteration of cytology of buccal cavity of tobacco users, leading to appearance of cytopathological features such as binucleation, karyorrhexis, karyolysis, pyknosis, and micronuclei.

Introduction

Tobacco use is the most important habit responsible for the cause of preventable and premature death globally, as it is a prime risk factor in the incidence of oral cancer (Rafaella *et al.,* 2021).

The World Health Organization estimates that tobacco kills about seven million people annually globally, and that an estimated 100 million deaths were recorded over the course of the 20th century (WHO, 2015).

Out of the almost 7million deaths, more than six million are the results of direct smoking (active smoking), while close to 900,000 deaths are the result of non-smokers exposed to second-hand smoke (passive smoking) (Vieira *et al.,* 2015). Unless urgent steps are taken, the annual death toll could rise to more than eight million by 2030 (Osibogun *et al.,* 2009).

Tobacco products contain chemicals that can be harmful to the health, and their smoke is a complex mixture of chemicals, most of which are carcinogenic to human (Herrington *et al.,* 2015).

Some of the chemicals found in tobacco smoke are Nicotine, Hydrogen cyanide, Benzene, Cadmium, Formaldehyde, Lead, Arsenic, ammonia, which are all inimical to the health. Almost all parts of the body are harmed by the tobacco smoke when inhaled actively or passively, as some of the health implications of tobacco use include cancers of the lungs, oral cavity, mouth, larynx, and oesophagus, heart disease, chronic obstructive pulmonary disease, and a host of others.

Oral cancer is one of the most common types of cancer throughout the world, and it is the sixth most common malignancy, being one of the major causes of cancer morbidity and mortality worldwide (Thambiah *et al.,* 2018). Tobacco use is an independent risk factor for the development of oral cancer, as its regular consumption is responsible for about 75% of all cases of Oral squamous cell carcinoma (Morrison, 2001).

Studies on tobacco use is still ongoing, but information is lacking on the use of other tobacco products like dried tobacco leaves used as snuff and chewing tobacco in Ibadan, Oyo state. Against this background, this study was conducted to determine the cytological changes in the oral mucosa of tobacco users in Ibadan, Oyo state, Nigeria.

Materials and Methods

Study Area

This study was conducted at different motor parks, drinking parlours and kiosks within Ibadan metropolis, Oyo State, southwestern Nigeria. Ibadan lies on the latitude 7.376736, and the longitude 3.939786**,** located in the cities place category with the GPS coordinates of 7° 22' 36.2496" N and 3° 56' 23.2296" E. The main economic activities engaged in by the Ibadan populace include agriculture, trade, public service employment, factory work, service sector/tertiary production.

Study Population

A total of 205 participants which comprised 140(68.3%) tobacco users and 65(31.7%) non users of tobacco were recruited for this study. A total number of 95(67.9%) males and 45(32.1%) females tobacco users and 35(53.8%) males and 30(46.2%) females non-tobacco users were used. In this study, tobacco user includes subjects that engage in tobacco usage in various forms, like smoke and smokeless tobacco products, whereas non-tobacco user includes those people who do not engage in the use of tobacco.

The age of these subjects ranged from 18 to 70years old. A well structured questionnaire was used to collect socio-demographic information from each participant prior to specimen collection.

Sample Collection and Handling

Buccal smear specimen was collected on a clean, grease-free slide using Ayre's spatula, thinned out and fixed in 95% ethanol. The slides were stained using Papanicolaou staining technique (Layton *et al.,* 2013). Fixed smears were hydrated and stained in Harris haematoxylin for 4 min, rinsed in water and differentiated in 0.5% acid alcohol briefly. Smears were rinsed in water and blued in tap water for 5min, and thereafter taken through 70% alcohol and two changes of 90% alcohol. Smears were stained in OG 6 solution for 2 min, rinsed in 2 changes of 95% alcohol and stained in EA 36 solution for 4 min. Stained smears were transferred into 2 changes of 95% alcohol, then 2 changes of absolute alcohol, cleared in xylene and mounted in DPX. The stained smears were viewed under microscope for cytomorphological appearances using X10 and X40 objective lenses.

Statistical Analysis

Statistical analysis of variables was carried out using Statistical Package for Social Science (SPSS 20.0). Prevalence of different cellular morphological appearance in tobacco users and non-users was tested using chi-square. The *p* values at less than 0.05 were considered significant.

Results and Discussion

Two hundred and five (205) participants were recruited, which comprised 140(68.3%) tobacco users and 65(31.7%) non-users of tobacco. From the 140 tobacco users, 95(67.9%) were male, while 45(32.1%) were females. From the 65 controls, 35(53.8%) were males, while 30(46.2%) were females.

The exfoliated buccal cells of tobacco users stained with Papanicolao showed varying shades of abnormalities, as indicated by the morphology of cell appearances. Cell morphology observed were binucleation, karyorrhexis, karyolysis, pyknosis, perinuclear halo, and micronucleus in the buccal smear of tobacco users.

From Table 1, Karyorrhexis (p=0.030), pyknosis (p=0.007), perinuclear halo (p=0.006) and binucleation (p=0.025) were significantly associated with tobacco users. However, karyolysis, and micronucleus did not significantly associate with tobacco users (p>0.05).

Many studies have correlated dysplasia and malignancy lesions in the oral mucosa with the use of different tobacco product, as all of the major forms of tobacco such as cigarettes, cigars, pipes, and smokeless tobacco (chewing tobacco and snuff) are known to cause oral cancer (Komal *et al.,* 2015).

It has been observed that tobacco and smokeless tobacco are capable of inducing malignant lesions in the oral mucosa of oral squamous cell carcinoma in city and county marked by increase in nuclear size has been reported from the smear of tobacco users (Saranya and Sudha, 2014). In this study, the abnormal cellular morphology reported were increased micronuclei, binucleation, karyorrhexis, karyolysis, pyknosis and perinuclear halo. The observations of these cell types in oral mucosa could be pointers to tumorigenesis. The increase in the frequency of these alterations is a viable index to assess the genotoxicity of exposure to various carcinogens by tobacco users.

Binucleation was significantly associated with tobacco users when compared with non-tobacco users (p= 0.025). This finding is consistent with that observed in the buccal smear of tobacco users in India (Sharma *et al.,* 2015). Kausar *et al.,* (2009) also reported the appearance of binucleation and other cellular abnormalties in a study conducted on smokeless tobacco users.

Also reported in a statistically significant number (p=0.029) is karyorrhexis, as these cells are mainly observed in the oral smear tobacco users. Karyorrhexis is a degenerative cellular process involving fragmentation of the nucleus and the breakup of the chromatin into unstructured granules (Komal *et al.,* 2015). There was an increase in karyorrhetic cells occurence in tobacco users compared with non-tobacco users in this study. This finding indicates the difference in mean karyorrhexis occurence of exfoliated buccal epithelial cells across the tobacco users and non-tobacco users was statistically higher in tobacco users (p=0.029). This report showed that tobacco users are at risk of significant cytogenic damage. This finding is in keeping with the report of the studies of Palaskara and Jindal (2010), and Borthakur *et al.,* (2008) that used Giemsa stain and observed a significant increase in the occurence of karyorrhexis and other nuclear abnormalities in the buccal cells of tobacco users than the non-users of tobacco.

In addition, micronucleation was reported as a cytopathological feature. Micronuclei are small membrane bounded compartments with a DNA content encapsulated by a nuclear envelope and spatially separated from the primary nucleus. Micronuclei (MNs) are extranuclear cytoplasmic DNA bodies which are induced in cells by numerous genotoxic agents that damage chromosome. They are induced in oral epithelium by a variety of substances including genotoxic agents and carcinogenic compounds in tobacco (Sarode *et al.,* 2015). Thomas *et al.,* (2009) reported that frequency of MN in oral mucosa cells of patients with OSCC was higher as compared with the control group. Khlifi *et al.,* (2013) observed a significantly elevated frequency of micronuclei (MN) and binucleated (BN) cells in the buccal mucosa of tobacco smokers when compared with non smokers. Khlifi *et al.,* (2013) also noted and reported a significant association of MN with tobacco smoking and chewing. Saranya and Sudha (2014) in a similar study also reported various cytomorphological changes including micronucleation in buccal mucosal epithelial cells of khaini (a product of tobacco) chewers in different age groups. Thomas *et al.,*

(2009) examined buccal mucosa of OSCC patients cells for genomic damage during exposure to tobacco products and a clear increase of MN was observed in the buccal mucosa. According to Singam *et al.,* (2019), the prevalence of MN in a healthy population was reportedly low and that the increased number of such structures would represent chromosomal aberrations. In the work of Upadhyay *et al.,* (2019) which sought to analyze the genotoxic effects of tobacco in individuals with a habit of smoking and chewing tobacco, the study reported a significant increase in the frequency of MN counting in individuals with a habit of smoking, chewing tobacco, or smoking and chewing tobacco concurrently, when compared to the control group, indicating that genotoxic and carcinogenic agents released by tobacco generate considerable genetic damage. Tomiazzi *et al.,* (2017) reported that the increased incidence of MN in exfoliated cells of the oral mucosa has been related to exposure to tobacco use.

In this study, there was no statistically difference in number of MN (p=0.335) in tobacco users compared with non-tobacco users (p=0.103), but numerically, the group of tobacco users presented with 0.93 compared with 0.25 of non-tobacco users. This difference in findings may be attributed to the fact that while OSCC confirmed patients were used in the reported studies, apparently healthy tobacco users were used in this study.

Perinuclear halo is a morphologic finding referring to the presence of a vacuolated area that surrounds the nucleus, resulting from nuclear shrinking. Perinuclear halo is the most common cytomorphologic change and is considered to be a major histopathologic feature for the determination of human papilloma virus (HPV) infection. A perinuclear halo is a squamous epithelial cell that has undergone a number of structural changes, which occur as a result of infection of the cell, and collectively, these types of changes are called a cytopathic effect. Presence of halo in oral smear is thought to represent a cytopathic effect of HPV, and is considered to give a clue to the diagnosis of HPV infection (Rakesh *et al.,* 2010). Presence of halo has been documented in smears and tissue sections of OSCC cases (Daniels *et al.,* 1992). In this study, perinuclear halo was observed in the smear of tobacco user in a significant number (x^2=7.403, p=0.06). The appearance of perinuclear halo which was only noticeable in tobacco users is suggesting that the use of tobacco also increases susceptibility to infection, as this is a cytopathological change found in infection such as HPV.

Karyolysis is the complete dissolution of the chromatin of a dying cell due to the enzymatic degradation by endonucleases. The whole cell will eventually stain uniformly with eosin after karyolysis. It is usually associated with karyorrhexis and occurs mainly as a result of necrosis, while in apoptosis after karyorrhexis the nucleus usually dissolves into apoptotic bodies. An increased number of KL cells has significance as these appear in the pre-keratinization process, which depicts an adaptive event to cellular trauma because of the chronic effects of the masticatory process in the oral mucosa (Komal *et al.,* 2015). In this study, karyolysis was observed in low significance (p=0.367).

The results obtained with the present study confirm the assumption that genetic changes may be induced by the presence of genotoxic agents from tobacco, which corroborates information previously reported in the literature. Tobacco usage has been linked to a wide variety of diseases, from superficial and easily treated lesions to more serious cancers of the oral mucous membranes (Göregen *et al.,* 2011). The appearance of many cytopathological anomalies reported in this study has provided a link between tobacco usage and oral malignancy. Oral mucosal epithelial cells proliferate more rapidly when exposed to heat and the chemical components of tobacco, therefore, the risk of developing OSCC is greater for tobacco users. Tobacco users without oral lesions, as seen in this study, have been studied using the Pap technique, which revealed higher rates of cellular proliferation in tobacco users as compared to non-tobacco users. This finding is in consistence with the previous report of Seifi *et al.,* (2013) that reported that tobacco consumers have a high risk of developing premalignant lesions with increase in tobacco volume used. In another study by Farhadi *et al.,* (2016) where effects of tobacco use on epithelial cells was examined, it was reported that tobacco users are more susceptible to nuclear alterations such as karyolysis, micronucei, and binucleation. Even in the absence of clinical lesions, the results of this study indicated that tobacco users have greater proliferative activity than non-tobacco users. This finding agrees with the report of Faris *et al.,* (2023) in a similar work where it was concluded that even if there are no clinical symptoms, tobacco users have been observed to have cytological changes like an increased nuclear-cytoplasmic ratio, irregular nuclear borders, inflammation, multi or bi-nucleation, increased keratinization, and changes in the shape and/or size of the cells and nuclei. This may be a sign of epithelial changes in response to a physiochemical environment.

Table.1 Association between cell morphology observable in tobacco and non-tobacco users

Morphology	$p-value$
N - Normal/Negative	0.001
CG - Cytoplasmic Granules	0.286
KH - Karyorrhesis	0.029
KL - Karyolysis	0.367
PN - Pyknosis	0.006
PH - Perinuclear Halo	0.006
BC - Binucleated Cell	0.024
MN - Micronucleus	0.335

*Significant at p< 0.05

The oral cytology screening was used in this study for representing a minimally invasive and low-cost method for studying cellular abnormalties in the oral cavity of tobacco users. Regarding the oral cavity, tobacco use has been described as the main risk factor for developing malignant and cancerous lesions (Sarode *et al.,* 2015), while the combination of alcoholism and tobacco use may increase the prevalence of oral cancer.

A greater aggressiveness of this disease in alcoholics and tobacco users may occur due to the increased permeability of the cell membrane caused by ethanol and consequent exposure of intracellular content to tobacco as a carcinogen (Mohammed and Mohammed, 2019). Based on the finding of this study, the tobacco users analyzed in this research do not yet present with clinical symptoms of neoplastic process, but have a markedly increased in the number of cellular anomalies observed.

In this study, the potential health risk of the tobacco users was evaluated through a genotoxic assay in exfoliated oral mucosa cells. In the results, the study showed that the tobacco use habit represented a significant factor for increasing the percentage of MN and other cellular anomalies.

Although it did not focus on assessing changes for tobacco use and drinking habits, this study however, also revealed that the tobacco users consumed alcoholic beverages concurrently. Tobacco use and alcohol consumption has been reported to have synergistic effect in initiating oral cancer, though the associated mechanism is not fully understood (Tauane *et al.,* 2021).

The incidence of carcinogenesis in the oral mucosa of smokers that consume alcohol is high, as alcohol acts as a solvent, and the cigarette, when in contact with the oral mucosa, releases toxins and thermal aggressions when lit (Vieira *et al.,* 2015). Thus, it causes a decrease in mucosal immunity and, consequently, provides the entry of carcinogenic agents present in tobacco into tissues (Rafaella *et al.,* 2021), increasing metabolism of carcinogenic substances.

Once oral and respiratory epithelial cells, which constitute the first physical barrier to genotoxic agents, become exposed to all mutagenic components of tobacco, alcohol, and other toxic chemicals, these exposed epithelial cells are excellent for monitoring and surveying for nuclear changes in them before the clinical symptoms of cancer appear.

The cellular alterations observed in this study can be attributed to the effect of the various byproducts (such as nitrosamine and nitrosonornicotine) released from tobacco use, that can influence cellular abnormalities. This observation agrees with the findings of Komal *et al.,* (2015) that in tobacco users, there is a close contact of the aforementioned tobacco byproducts, thus facilitating their infiltration into the mucosa, which may be responsible for the noticeable cellular changes.

The smears of tobacco users revealed increased micronuclei, binucleation, karyorrhexis, karyolysis and perinuclear halos. From the foregoing, it can be safely concluded that tobacco use greatly caused alteration of cytology of the buccal cavity of tobacco users.

Availability of data and material

The data and materials supporting the findings of this research work are available on request from the authors.

Authors' Contribution

Trimiziy Kehinde Olatunji conceived the research idea, assisted in slide screening and writing the manuscript. Frederick Olusegun Akinbo assisted in writing and reviewing the manuscript.

Acknowledgements

The authors will like to acknowledge our respective families and MLS colleagues at the Pathology department of the University College Hospital, Ibadan for their assistance in slide processing. The management of National Veterinary Research Institute, Vom, Nigeria is deeply appreciated for the success of this research work.

Funding: This work was self funded by the authors.

Declarations

Ethics approval and consent to participate: The protocol for this study was approved by the Ethics and Research Committee of the Ministry of Health, Oyo State, Nigeria. Informed consent was sought from each participant through writing before the collection of specimen.

Consent for publications: All the authors involved in this work gave consent to its publication.

Conflict of Interest The authors declare no competing interests.

Individual video/data consent for publication: Not applicable.

Competing interest: the authors declare no known competing interest that could influence the objectivity of this publication.

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How to cite this article:

Trimiziy Kehinde Olatunji and Frederick Olusegun Akinbo. 2025. Cytopathological Changes in the Oral Mucosa of Tobacco Users in Ibadan, Nigeria. *Int.J.Curr.Microbiol.App.Sci.* 14(06): 293-300.
doi: https://doi.org/10.20546/ijcmas.2025.1406.026